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amended

introducing a DNA fragment into a plant cell, wherein the DNA fragment comprises a selectable marker;
growing the cell under selective conditions to produce cells that have incorporated the DNA fragment into their genomic DNA;
selecting from among those cells a cell that has produced a dicentric chromosome; and
growing that cell under selective conditions, whereby a cell that contains a chromosome comprising a heterochromatic arm is produced.

58 59. A method of producing a transgenic plant, comprising:
introducing a DNA fragment into a first cell, wherein the DNA fragment comprises a selectable marker;
growing the cell under selective conditions to produce cells that have incorporated the DNA fragment into their genomic DNA; and
selecting a cell that comprises a satellite artificial chromosome (SATAC); and
isolating the SATAC and introducing it into a plant cell.

REMARKS

Any fees that may be due in connection with this application throughout its pendency may be charged to Deposit Account No. 50-1213.

Claim 43 is amended to correct a punctuation error. A colon was added after "comprising." Claim 59 is amended to remove the reference to "animal" since the claim is directed to a method for transgenic plant production.

The specification is amended to correct obvious typographical, spelling and formatting errors. No new matter has been added to the claims or the specification.

In view of the amendments and above remarks, entry of the amendments and examination of the application on the merits are respectfully requested.

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Respectfully submitted,
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: HADLACZKY *et al.*

Serial No. 09/724,726

Filed: November 28, 2000

For: *ARTIFICIAL CHROMOSOMES, USES
THEREOF AND METHODS FOR
PREPARING ARTIFICIAL
CHROMOSOMES*

Art Unit: Unassigned

Examiner: Unassigned

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05/14/01

Date

Kelly M. Fischer
Kelly M. Fischer

**ATTACHMENT TO THE PRELIMINARY AMENDMENT
MARKED UP PARAGRAPHS AND CLAIMS (37 CFR §1.121)**

IN THE SPECIFICATION:

Please amend the specification as follows:

Please amend the paragraphs on page 19, lines 4-15, as follows:

As used herein, a SATAC refers to a chromosome that is substantially all heterochromatin, except for portions of heterologous DNA. Typically, SATACs are satellite DNA based artificial chromosomes, but the term [encompasses] encompasses any chromosome made by the methods herein that contains more heterochromatin than euchromatin.

As used herein, amplifiable, when used in reference to a chromosome, particularly the method of generating SATACs provided herein, refers to a region of a chromosome that is prone to amplification. [Amplification] Amplification typically occurs during replication and other cellular events involving recombination. Such regions are typically regions of the chromosome that include tandem repeats, such as satellite DNA, rDNA and other such sequences.

Please amend the paragraph on page 23, line 18, to page 24, line 11, as follows:

As used herein, transformation/transfection refers to the process by which DNA or RNA is introduced into cells. Transfection refers to the taking up

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of exogenous nucleic acid, e.g., an expression vector, by a host cell whether or not any coding sequences are in fact expressed. Numerous methods of transfection are known to the ordinarily skilled artisan, for example, by direct uptake using calcium phosphate (CaPO₄; see, e.g., Wigler *et al.* (1979) *Proc. Natl. Acad. Sci. U.S.A.* 76:1373-1376), polyethylene glycol (PEG)-mediated DNA uptake, electroporation, lipofection (see, e.g., Strauss (1996) *Meth. Mol. Biol.* 54:307-327), microcell fusion (see, EXAMPLES, see, also Lambert (1991) *Proc. Natl. Acad. Sci. U.S.A.* 88:5907-5911; U.S. Patent No. 5,396,767, [Sawford] Sanford *et al.* (1987) *Somatic Cell Mol. Genet.* 13:279-284; Dhar *et al.* (1984) *Somatic Cell Mol. Genet.* 10:547-559; and McNeill-Killary *et al.* (1995) *Meth. Enzymol.* 254:133-152), lipid-mediated carrier systems (see, e.g., Teifel *et al.* (1995) *Biotechniques* 19:79-80; Albrecht *et al.* (1996) *Ann. Hematol.* 72:73-79; Holmen *et al.* (1995) *In Vitro Cell Dev. Biol. Anim.* 31:347-351; [REmy] Remy *et al.* (1994) *Bioconjug. Chem.* 5:647-654; Le Bolch *et al.* (1995) *Tetrahedron Lett.* 36:6681-6684; Loeffler *et al.* (1993) *Meth. Enzymol.* 217:599-618) or other suitable method. Successful transfection is generally recognized by detection of the presence of the heterologous nucleic acid within the transfected cell, such as any indication of the operation of a vector within the host cell. Transformation means introducing DNA into an organism so that the DNA is replicable, either as an extrachromosomal element or by chromosomal integration.

Please amend the paragraph on page 27, line 22, to page 28, line 2, as follows:

DNA sequences that provide a preferred megareplicator are the rDNA units that give rise to ribosomal RNA (rRNA). In mammals, particularly mice and humans, these rDNA units contain specialized elements, such as the origin of replication (or origin of bidirectional replication, i.e., OBR, in mouse) and amplification promoting sequences (APS) and amplification control elements (ACE) (see, e.g., Gogel *et al.* (1996) *Chromosoma* 104:511-518; Coffman *et al.* (1993) *Exp. Cell. Res.* 209:123-132; Little *et al.* (1993) *Mol. Cell. Biol.*

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13:6600-6613; Yoon *et al.* (1995) *Mol. Cell. Biol.* 15:2482-2489; Gonzalez and Sylvester (1995) *Genomics* 27:320-328; Miesfeld and Arnheim (1982) *Nuc. Acids Res.* 10:3933-3949[[]]; Maden *et al.* (1987) *Biochem. J.* 246:519-527).

Please amend the paragraph on page 36, lines 3-15, as follows:

As shown herein, this phenomenon is generalizable and can be observed with other chromosomes. Also, although these *de novo* formed chromosome segments and chromosomes appear different, there are similarities that indicate that a similar amplification mechanism plays a role in their formation: (i) in each case, the amplification is initiated in the centromeric region of the mouse chromosomes and large (Mb size) amplicons are formed; (ii) mouse major satellite DNA sequences are constant constituents of the amplicons, either by providing the bulk of the heterochromatic amplicons (H-type amplification), or by bordering the [a]euchromatic amplicons (E-type amplification); (iii) formation of inverted segments can be demonstrated in the λ neo-chromosome and megachromosome; (iv) chromosome arms and chromosomes formed by the amplification are stable and functional.

Please amend the paragraph on page 37, lines 14-23, as follows:

Introduction of additional heterologous DNA, including DNA encoding a second selectable marker, hygromycin phosphotransferase, i.e., the hygromycin-resistance gene, and also a detectable marker, β -galactosidase (i.e., encoded by the lacZ gene), into the EC3/7C5 cell line and growth under selective conditions produced cells designated TF1004G19. In particular, this cell line was produced from the EC3/7C5 cell line by cotransfection with plasmids pH132, which contains an anti-HIV ribozyme and hygromycin-resistance gene, pCH110 (encodes β -galactosidase) and λ phage (λ cl [875] 857 Sam 7) DNA and selection with hygromycin B.

Please amend the paragraph on page 66, line 20, to page 67, line 15 as follows:

Because there is a large variety of restriction enzymes that recognize many different DNA sequences as cleavage sites, it should always be possible

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to select sites and enzymes (preferably those that yield a 3'-protruding end) suitable for these methods in connection with the synthesis of any one particular repeat [array] array. In most cases, only 1 (or perhaps 2) nucleotide(s) has of a restriction site is required to be present in the repeat sequence, and the remaining nucleotides of the restriction site can be removed, for example:

PacI: TTAAT/TAA-- (Klenow/dNTP) TAA--

PstI: CTGCA/G-- (Klenow/dNTP) G--

NsiI: ATGCA/T-- (Klenow/dNTP) T--

KpnI: GGTAC/C-- (Klenow/dNTP) C--

Though there is no known restriction enzyme leaving a single A behind, this problem can be solved with enzymes leaving behind none at all, for example:

TaII: ACGT/ (Klenow/dNTP) --

NlaIII: CATG/ (Klenow/dNTP) --

Additionally, if mung bean nuclease is used instead of Klenow, then the following:

XbaI: T/CTAGA Mung bean nuclease A--

Furthermore, there are a number of restriction enzymes that cut outside of the recognition sequence, and in this case, there is no limitation at all:

TspRI NNCAGTGNN/-- (Klenow/dNTP) --

BsmI GAATG CN/-- (Klenow/dNTP) --

CTTAC/GN -- (Klenow/dNTP) --

Please amend the paragraph on page 71, line 19 to page 72, line 2, as follows:

The microcells prepared from the 1B3 and GHB42 cells were fused with E2D6K. The E2D6K cells were generated by CaPO₄ transfection of CHO K-20 cells with pCHTV2. Plasmid pCHTV2 contains the puromycin-resistance gene

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linked to the SV40 promoter and polyadenylation signal, the *Saccharomyces cerevisiae* URA3 gene, 2.4- and 3.2-kb fragments of a Chinese hamster chromosome 2-specific satellite DNA (HC-2 satellite; see Fatyol *et al.* (1994) *Nuc. Acids Res.* 22:3728-3736), two copies of the [diphtheria] diphtheria toxin-A chain gene (one linked to the herpes simplex virus thymidine kinase (HSV-TK) gene promoter and SV40 polyadenylation signal and the other linked to the HSV-TK promoter without a polyadenylation signal), the ampicillin-resistance gene and the ColE1 origin of replication. Following transfection, puromycin-resistant colonies were isolated. The presence of the pCHTV2 plasmid in the E2D6K cell line was confirmed by nucleic acid amplification of DNA isolated from the cells.

Please amend the paragraph on page 74, lines 7-9, as follows:

For cotransfection and for *in situ* hybridization, the pCH110 β -galactosidase construct (Pharmacia or Invitrogen), and λ cl [875] 857 Sam7 phage DNA (New England Biolabs) were used.

Please amend the paragraph on page 74, line 23, to page 75, line 7, as follows:

Cell lines KE1-2/4, EC3/7C5, TF1004G19C5, 19C5xHa4, G3D5 and H1D3 have been deposited in accord with the Budapest Treaty at the European Collection of Animal Cell Culture (ECACC) under Accession Nos. 96040924, 96040925, 96040926, 96040927, 96040928 and 96040929, respectively. The cell lines were deposited on April 9, 1996, at the European Collection of Animal Cell Cultures (ECACC) Vaccine Research and Production Laboratory, Public Health Laboratory Service, Centre for [Applied] Applied Microbiology and Research, Porton Down, Salisbury, Wiltshire SP4 0JG, United Kingdom. The deposits were made in the name of Gyula Hadlaczky of H. 6723, SZEGED, SZAMOS U.1.A. IX. 36. HUNGARY, who has authorized reference to the deposited cell lines in this application.

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Please amend the paragraph on page 86, lines 9-14, as follows:

Cotransfection of EC3/7C5 cells with plasmids (pH132, pCH110 available from Pharmacia, see, also Hall *et al.* (1983) *J. Mol. Appl. Gen.* 2:101-109) and with λ DNA (λ cl [875] 857 Sam 7(New England Biolabs)) was conducted using the calcium phosphate DNA precipitation method (see, *e.g.*, Chen *et al.* (1987) *Mol. Cell. Biol.* 7:2745-2752), using 2-5 μ g plasmid DNA and 20 μ g λ phage DNA per 5×10^6 recipient cells.

Please amend the paragraph on page 90, line 1-9, as follows:

To promote further elimination of mouse chromosomes, hybrid cells were repeatedly treated with BrdU. The BrdU treatments, which destabilize the genome, result in significant loss of mouse chromosomes. The BrdU-treated 19C5xHa4 hybrid cells were divided to three groups. One group of the hybrid cells (GH) [were] was maintained in the presence of hygromycin (200 μ g/ml) and G418 (400 μ g/ml), and the other two groups of the cells were cultured under G418 (G) or hygromycin (H) selection conditions to promote the elimination of the sausage chromosome or minichromosome.

Please amend the paragraphs on page 98, line 3, to page 99, line 4, as follows:

Large-scale mapping of the megachromosome around the area of the site of integration of the heterologous DNA revealed that it is enriched in sequence containing rare-cutting enzyme sites, such as the recognition site for *NotI*. Additionally, mouse major satellite DNA (which makes up the majority of the megachromosome) does not contain *NotI* recognition sites. Therefore, to facilitate isolation of regions of the megachromosome associated with the site of integration of the heterologous DNA, the isolated megachromosomes were cleaved with *NotI*, a rare cutting restriction endonuclease with an 8-bp GC recognition site. Fragments of the megachromosome were inserted into plasmid pWE15 (Stratagene, La Jolla, California) as follows. Half of a 100- μ l low melting point agarose block (mega-plug) containing the isolated SATACs was digested with *NotI* overnight at 37°C. Plasmid pWE15 was similarly digested

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with *NotI* overnight. The mega-plug was then melted and mixed with the digested plasmid, ligation buffer and T4 ligase. Ligation was conducted at 16°C overnight. Bacterial DH5 α cells were transformed with the ligation product and transformed cells were plated onto LB/Amp plates. Fifteen to twenty colonies were grown on each plate for a total of 189 colonies. Plasmid DNA was isolated from colonies that survived growth on LB/Amp medium and was analyzed by Southern blot hybridization for the presence of DNA that hybridized to a pUC19 probe. This screening methodology assured that all clones, even clones lacking an insert but yet containing the pWE15 plasmid, would be detected. Any clones containing insert DNA would be expected to contain [contain] non-satellite, GC-rich megachromosome DNA sequences located at the site of integration of the heterologous DNA. All colonies were positive for hybridizing DNA.

Liquid cultures of all 189 transformants were used to generate cosmid minipreps for analysis of restriction sites within the insert DNA. Six of the original 189 cosmid clones [conatained] contained an insert. These clones were designated as follows: 28 (~9-kb insert), 30 (~9-kb insert), 60 (~4-kb insert), 113 (~9-kb insert), 157 (~9-kb insert) and 161 (~9-kb insert). Restriction enzyme analysis indicated that three of the clones (113, 157 and 161) contained the same insert.

Please amend the table on page 99 to page 100 as follows:

CELL TYPE	PROBE	LOCATION OF SIGNAL
Human Lymphocyte (male)	No. 161	4-5 pairs of [acrocentric] <u>acrocentric</u> chromosomes at centromeric regions.
Mouse Spleen	No. 161	Acrocentric ends of 4 pairs of chromosomes.
EC3/7C5 Cells	No. 161	Minichromosome and the end of the formerly dicentric chromosome. Pericentric heterochromatin of one of the metacentric mouse chromosomes. Centromeric region of some of the other mouse chromosomes.

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CELL TYPE	PROBE	LOCATION OF SIGNAL
K20 Chinese Hamster Cells	No. 30	Ends of at least 6 pairs of chromosomes. An interstitial signal on a short chromosome.
HB31 Cells (mouse-hamster hybrid cells derived from H1D3 cells by repeated BrdU treatment and single cell cloning which carries the megachromosome)	No. 30	Acrocentric ends of at least 12 pairs of chromosomes. Centromeres of certain chromosomes and the megachromosome. Borders of the amplicons of the megachromosome.
Mouse Spleen Cells	No. 30	Similar to signal observed for probe no. 161. Centromeres of 5 pairs of chromosomes. Weak cross- hybridization to pericentric heterochromatin.
HB31 Cells	No. 113	Similar to signal observed for probe no. 30.
Mouse Spleen Cells	No. 113	Centromeric region of 5 pairs of chromosomes.
K20 Cells	No. 113	At least 6 pairs of chromosomes. Weak signal at some telomeres and several interspersed signals.
Human Lymphocyte Cells (male)	No. 157	Similar to signal observed for probe no. 161.

Please amend the paragraph on page 103, line 27, to page 104 line 4, as follows:

Heterologous gene expression levels of H1D3 cells, carrying a 250-400 Mb megachromosome as [decribed] described above, and mM2C1 cells, carrying a 50-60 Mb micro-megachromosome, were quantitatively evaluated. mM2C1 cells were generated by repeated BrdU treatment and single cell cloning of the H1xHe41 cell line (mouse-hamster-human hybrid cell line carrying the megachromosome and a single human chromosome with CD4 and neo^r genes; see Figure 4). The cell lines were grown under standard conditions in F12 medium under selective (120 μ g/ml hygromycin) or non-selective conditions.

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Please amend the paragraphs on page 105, line 2 to page 106, line 18, as follows:

Enzyme activity was measured by means of the phosphocellulose paper binding assay as described by Haas and Dowding ((1975). *Meth. Enzymol.* 43:611-628). The cell extract was supplemented with 0.1 M ammonium chloride and 1 mM adenosine- γ -³²P-triphosphate (specific activity: 300 Ci/mmol). The reaction was initiated by the addition of 0.1 mg/ml hygromycin and incubated for increasing time at 37°C. The reaction was terminated by heating the samples for 5 min at 75°C in a water bath, and after removing the precipitated proteins by centrifugation for 5 min in a microcentrifuge, an aliquot of the supernatant was spotted on a piece of Whatman P-81 phosphocellulose paper (2 cm²). After 30 sec at room temperature the papers are placed into 500 ml of hot (75°C) distilled water for 3 min. While the radioactive ATP remains in solution under these conditions, hygromycin phosphate binds strongly and quantitatively to phosphocellulose. The papers are rinsed 3 times in 500 ml of distilled water and the bound radioactivity was measured in toluene scintillation cocktail in a Beckman liquid scintillation counter. Reaction mixture incubated without added hygromycin served as a control.

f. Determination of the copy-number of the heterologous genes

DNA was prepared from the H1D3 and mM2C1 cells using standard purification protocols involving SDS lysis of the cells followed by Proteinase K treatment and phenol/chloroform extractions. The isolated DNA was digested with an appropriate restriction endonuclease, fractionated on agarose gels, blotted to nylon filters and hybridized with a radioactive probe derived either from the β -galactosidase or the hygromycin phosphotransferase genes. The level of hybridization was quantified in a Molecular Dynamics PhosphorImage Analyzer. To control the total amount of DNA loaded from the different cell lines, the filters were reprobated with a single copy gene, and the [hybridization] hybridization of β -galactosidase and hygromycin phosphotransferase genes was normalized to the single copy gene hybridization.

g. Determination of protein concentration

The total protein content of the cell extracts was measured by the Bradford colorimetric assay using bovine serum albumin as standard.

2. Characterization of the β -galactosidase and hygromycin phosphotransferase activity expressed in H1D3 and mM2C1 cells

In order to establish [quantative] quantitative conditions, the most important kinetic parameters of β -galactosidase and hygromycin phosphotransferase activity have been studied. The β -galactosidase activity measured with a colorimetric assay was linear between the 0.1-0.8 OD₄₂₀ range both for the mM2C1 and H1D3 cell lines. The β -galactosidase activity was also proportional in both cell lines with the amount of protein added to the reaction mixture within 5-100 μ g total protein concentration range. The hygromycin phosphotransferase activity of mM2C1 and H1D3 cell lines was also proportional with the reaction time or the total amount of added cell extract under the conditions described for the β -galactosidase.

Please amend the paragraph on page 108, lines 8-28, as follows:

Quantitative determination of β -galactosidase activity of higher eukaryotic cells (*e.g.*, H1D3 cells) carrying the bacterial β -galactosidase gene in heterochromatic megachromosomes confirmed the observed high-level expression of the integrated bacterial gene detected by cytological staining methods. It has generally been established in reports of studies of the expression of foreign genes in transgenic animals that, although transgene expression shows correct tissue and developmental specificity, the level of expression is typically low and shows extensive position-dependent [variability] variability (*i.e.*, the level of transgene expression depends on the site of chromosomal integration). It has been assumed that the low-level transgene expression may be due to the absence of special DNA sequences which can insulate the transgene from the inhibitory effect of the surrounding chromatin and promote the formation of active chromatin structure required for efficient gene expression. Several *cis*-[activating] activating DNA sequence elements have

been identified that abolish this position-dependent variability, and can ensure high-level expression of the transgene locus [activating] activating region (LAR) sequences in higher eukaryotes and specific chromatin structure (scs) elements in lower eukaryotes (see, *et al.* Eissenberg and Elgin (1991) *Trends in Genet.* 7:335-340). If these cis-acting DNA sequences are absent, the level of transgene expression is low and copy-number independent.

Please amend the paragraph on page 110, line 31, to page 111 line 8, as follows:

The LMTK⁻-derived cell line, which is a mouse fibroblast cell line, was transfected with λ CM8 and λ gtWESneo DNA (see, EXAMPLE 2) to produce transformed cell lines. Among these, [was] was EC3/7, deposited at the European Collection of Animal cell Culture (ECACC) under Accession No. 90051001 (see, U.S. Patent No. 5,288,625; see, also Hadlaczký *et al.* (1991) *Proc. Natl. Acad. Sci. U.S.A.* 88:8106-8110 and U.S. application Serial No. 08/375,271). This cell line contains the dicentric chromosome with the neo-centromere. Recloning and selection produced cell lines such as EC3/7C5, which are cell lines with the stable neo-minichromosome and the formerly dicentric chromosome (see, Fig. 2C).

Please amend the paragraphs on page 111, line 27, to page 112, line 19, as follows:

As discussed above, cotransfection of EC3/7C5 cells with plasmids (pH132, pCH110 available from Pharmacia, see, also Hall *et al.* (1983) *J. Mol. Appl. Gen.* 2:101-109) and with λ DNA (λ cl [875] 857 Sam 7 (New England Biolabs)) produced transformed cells. Among these is TF1004G24, which contains the DNA encoding the anti-HIV ribozyme in the neo-minichromosome. Recloning of TF1004G24 produced numerous cell lines. Among these is the NHHL24 cell line. This cell line also has the anti-HIV ribozyme in the neo-minichromosome and expresses high levels of β -gal. It has been fused with CHO-K20 cells to produce various hybrids.

5. TF1004G19-Derived cells

Recloning and selection of the TF1004G transformants produced the cell line TF1004G19, discussed above in EXAMPLE 4, which contains the unstable sausage chromosome and the neo-minichromosome. Single cell cloning produced the TF1004G-19C5 (see Figure 4) cell line, which has a stable sausage chromosome and the neo-[minichromosome] minichromosome. TF1004G-19C5 has been fused with CHO cells and the hybrids grown under selective conditions to produce the 19C5xHa4 and 19C5xHa3 cell lines (see, EXAMPLE 4) and others. Recloning of the 19C5xHa3 cell line yielded a cell line containing a gigachromosome, i.e., cell line 19C5xHa47, see Figure 2E. BrdU treatment of 19C5xHa4 cells and growth under selective conditions (neomycin (G) and/or hygromycin (H)) has produced hybrid cell lines such as the G3D5 and G4D6 cell lines and others. G3D5 has the neo-minichromosome and the megachromosome. G4D6 has only the neo-minichromosome.

Please amend the paragraph on page 114, lines 27-29, as follows:

All observations and microphotography were made by using a Vanox AHBS (Olympus) microscope. Fujicolor 400 Super G or Fujicolor 1600 Super HG high-speed [colour] color negatives were used for photographs.

Please amend the paragraphs on page 124, line 6 to page 126, line 8 as follows:

Artificial chromosomes, such as SATACs, may be sorted from endogenous chromosomes using any suitable procedures, and typically involve isolating metaphase chromosomes, distinguishing the artificial chromosomes from the endogenous chromosomes, and separating the artificial chromosomes from endogenous chromosomes. Such procedures will generally include the following basic steps: (1) culture of a sufficient number of cells (typically about 2×10^7 mitotic cells) to yield, preferably on the order of 1×10^6 artificial chromosomes, (2) arrest of the cell cycle of the cells in a stage of mitosis, [preferably] preferably metaphase, using a mitotic arrest agent such as colchicine, (3) treatment of the cells, particularly by swelling of the cells in hypotonic buffer, to increase susceptibility of the cells to disruption, (4) by

application of physical force to disrupt the cells in the presence of isolation buffers for stabilization of the released chromosomes, (5) dispersal of chromosomes in the presence of isolation buffers for stabilization of free chromosomes, (6) separation of artificial from endogenous chromosomes and (7) storage (and shipping if desired) of the isolated artificial chromosomes in appropriate buffers. Modifications and variations of the general procedure for isolation of artificial chromosomes, for example to accommodate different cell types with differing growth characteristics and requirements and to optimize the duration of mitotic block with arresting agents to obtain the desired balance of chromosome yield and level of debris, may be empirically determined.

Steps 1-5 relate to isolation of metaphase chromosomes. The separation of artificial from endogenous chromosomes (step 6) may be accomplished in a variety of ways. For example, the chromosomes may be stained with DNA-specific dyes such as Hoeschst 33258 and chromomycin A₃ and sorted into artificial and endogenous chromosomes on the basis of dye content by employing fluorescence-activated cell sorting (FACS). To facilitate larger scale isolation of the artificial chromosomes, different separation [techniques] techniques may be employed such as swinging bucket centrifugation (to effect separation based on chromosome size and density) (see, e.g., Mendelsohn *et al.* (1968) *J. Mol. Biol.* 32:101-108), zonal rotor centrifugation (to effect separation on the basis of chromosome size and density) (see, e.g., Burki *et al.* (1973) *Prep. Biochem.* 3:157-182; Stubblefield *et al.* (1978) *Biochem. Biophys. Res. Commun.* 83:1404-1414, velocity sedimentation (to effect separation on the basis of chromosome size and shape) (see e.g., Collard *et al.* (1984) *Cytometry* 5:9-19). Immuno-affinity purification may also be employed in larger scale artificial chromosome isolation procedures. In this process, large populations of artificial chromosome-containing cells (asynchronous or mitotically enriched) are harvested en masse and the mitotic chromosomes (which can be released from the cells using standard procedures such as by incubation of the cells in hypotonic buffer and/or detergent treatment of the cells in conjunction with

physical disruption of the treated cells) are enriched by binding to antibodies that are bound to solid state matrices (e.g. column resins or magnetic beads). Antibodies suitable for use in this procedure bind to condensed centromeric proteins or condensed and DNA-bound histone proteins. For example, autoantibody LU851 (see Hadlaczký *et al.* (1989) *Chromosoma* 97:282-288), which recognizes mammalian centromeres may be used for large-scale isolation of chromosomes prior to subsequent separation of artificial from endogenous chromosomes using methods such as FACS. The bound chromosomes would be washed and eventually eluted for sorting. Immunoaffinity purification may also be used directly to separate artificial chromosomes from endogenous chromosomes. For example, SATACs may be generated in or transferred to (e.g., by microinjection or microcell fusion as described herein) a cell line that has chromosomes that contain relatively small amounts of heterochromatin, such as hamster cells (e.g., V79 cells or CHO-K1 cells). The SATACs, which are predominantly heterochromatin, are then separated from the endogenous chromosomes by utilizing anti-heterochromatin binding protein (*Drosophila* HP-1) antibody conjugated to a solid matrix. Such matrix preferentially binds SATACs relative to hamster chromosomes. Unbound hamster chromosomes are washed away from the matrix and the SATACs are eluted by standard techniques.

Please amend the paragraph on page 128, lines 6-19, as follows:

Accordingly, several considerations may go into the selection of host cells for the production and isolation of artificial chromosomes. It may be that the host cell selected as the most desirable for *de novo* formation of artificial chromosomes is not optimized for large-scale production of the artificial chromosomes generated in the cell line. In such cases, it may be possible, once the artificial chromosome has been generated in the initial host cell line, to transfer it to a production cell line more well suited to efficient, high-level production and isolation of the artificial chromosome. Such transfer may be accomplished through several methods, for example through microcell fusion, as described herein, or microinjection into the production cell line of artificial

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chromosomes purified from the generating cell line using procedures such as described herein. Production cell lines preferably contain two or more copies of the [artificial] artificial chromosome per cell.

Please amend the paragraph on page 129, lines 3-28, as follows:

Cells (grown on plastic or in suspension) may be arrested in different stages of the cell cycle with chemical agents other than colchicine, such as hydroxyurea, vinblastine, colcemid or aphidicolin. Chemical agents that arrest the cells in stages other than mitosis, such as hydroxyurea and aphidicolin, are used to synchronize the cycles of all cells in the population and then are removed from the cell medium to allow the cells to proceed, more or less simultaneously, to mitosis at which time they may be harvested to disperse the chromosomes. Mitotic cells could be enriched for a mechanical shake-off (adherent cells). The cell cycles of cells within a population of MAC-containing cells may also be synchronized by nutrient, growth factor or hormone deprivation which leads to an accumulation of cells in the G₁ or G₀ stage; readdition of nutrients or growth factors then allows the quiescent cells to re-enter [the] the cell cycle in synchrony for [abot] about one generation. Cell lines that are known to respond to hormone deprivation in this manner, and which are suitable as hosts for artificial chromosomes, include the Nb2 rat lymphoma cell line which is absolutely dependent on prolactin for stimulation of proliferation (see Gout *et al.* (1980) *Cancer Res.* 40:2433-2436). Culturing the cells in prolactin-deficient medium for 18-24 hours leads to arrest of proliferation, with cells accumulating early in the G₁ phase of the cell cycle. Upon addition of prolactin, all the cells progress through the cell cycle until M phase at which point greater than 90% of the cells would be in mitosis (addition of colchicine could increase the amount of the mitotic cells to greater than 95%). The time between reestablishing proliferation by prolactin addition and harvesting mitotic cells for chromosome separation may be empirically determined.

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Please amend the paragraph on page 137, lines 10-13, as follows:

2. The large size of an artificial chromosome permits megabase size DNA integration so that genes encoding an entire pathway leading to a protein or nonprotein of therapeutic value, such as an alkaloid (digitalis, morphine, taxol) can be [accomodated] accommodated by the artificial chromosome.

Please amend the paragraph on page 142, lines 4-19, as follows:

To generate a synthetic telomere made up of multiple repeats of the sequence TTAGGG, attempts were made to clone or amplify ligation products of 30-mer oligonucleotides containing repeats of the sequence. Two 30-mer oligonucleotides, one containing four repeats of TTAGGG bounded on each end of the complete run of repeats by half of a repeat and the other containing five repeats of the complement AATCCC, were annealed. The resulting double-[standed] stranded molecule with 3-bp protruding ends, each representing half of a repeat, was expected to ligate with itself to yield concatamers of $n \times 30$ bp. However, this approach was unsuccessful, likely due to formation of quadruplex DNA from the G-rich strand. Similar difficulty has been encountered in attempts to generate long repeats of the pentameric human satellite II and III units. Thus, it appears that, in general, any oligomer sequence containing periodically spaced consecutive series of guanine nucleotides is likely to form undesired quadruplex formation that hinders construction of long double-stranded DNAs containing the sequence.

Please amend the paragraph on page 152, line 23, to page 153, line 12, as follows:

Plasmid pUP-CFTR was then linearized by partial digestion with *EcoRI* and the 13 kb fragment containing the CFTR gene was ligated with *EcoRI*-digested Charon 4A λ (see Blattner *et al.* (1977) *Science* 196:161; Williams and Blattner (1979) *J. Virol.* 29:555 and Sambrook *et al.* (1989) *Molecular Cloning, A Laboratory Manual*, Second Ed., Cold Spring Harbor Laboratory Press, Volume 1, Section 2.18, for descriptions of Charon 4A λ). The resulting vector, λ CF8, contains the Charon 4A λ bacteriophage left arm, the CFTR gene linked to the

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CMV promoter and SV40 polyadenylation signal, the *ura3* gene, the puromycin-resistance gene linked to the SV40 promoter and polyadenylation signal, the thymidine kinase promoter (TK), the ColE1 origin of [replicaton] replication, the [ampicillan] ampicillin resistance gene and the Charon 4A λ bacteriophage right arm. The λ CF8 construct was then digested with *Xho*I and the resulting 27.1 kb was ligated to the 0.4kb *Xho*I/*Eco*RI fragment of pJBP86 (described below), containing the SV40 polyA signal and the *Eco*RI-digested Charon 4A λ right arm. The resulting vector λ CF-7 contains the Charon 4A λ left arm, the CFTR encoding DNA linked to the CMV promoter and SV40 polyA signal, the *ura3* gene, the puromycin resistance gene linked to the SV40 promoter and polyA signal and the Charon 4A λ right arm. The λ DNA fragments provide encode sequences homologous to nucleotides present in the exemplary artificial chromosomes.

Please amend the paragraph on page 153, lines 20-23, as follows:

Vector λ CF-7-DTA also contains all the elements contained in λ CF-7, but additionally contains a lethal selection marker, the [diphtheria] diphtheria toxin-A (DT-A) gene as well as the ampicillin-resistance gene and an origin of replication. This vector was constructed in a series of steps as follows.

Please amend the paragraph on page 154, lines 7-18 as follows:

A 1.1-kb *Xho*I/*Sa*I fragment of pMC1-DT-A (see, *e.g.*, Maxwell *et al.* (1986) *Cancer Res.* 46:4660-4666) containing the [diphtheria] diphtheria toxin-A gene was ligated to *Xho*I-digested pMEP4 (Invitrogen, San Diego, CA) to generate pMEP-DTA. To produce pMC1-DT-A, the coding region of the DTA gene was isolated as a 800 bp *Pst*I/*Hind*III fragment from p2249-1 and inserted into pMC1neopolyA (pMC1 available from Stratagene) in place of the neo gene and under the control of the TK [promotoer] promoter. The resulting construct pMC1DT-A was digested with *Hind*III, the ends filled by Klenow and *Sa*I linkers were ligated to produce a 1061 bp TK-DTA gene cassette with an *Xho*I end (5') and a *Sa*I end containing the 270 bp TK promoter and the ~790 bp DT-A fragment. This fragment was ligated into *Xho*I-digested pMEP4 .

Please amend the paragraph on page 155, lines 2-9, as follows:

The 12-bp overhangs of λ CF-7 were removed by Mung bean nuclease and subsequent T4 polymerase treatments. The resulting 41.1-kb linear λ CF-7 vector was then ligated to pFB83-DTA9 which had been digested with *Cla*I and treated with T4 polymerase. The resulting vector, λ CF-7-DTA, contains all the elements of λ CF-7 as well as the DT-A gene linked to the TK promoter and the SV40 polyadenylation signal, the 1.8 kB Charon 4A λ COS region, the [ampicillin] ampicillin-resistance gene (from pJB83-DTA9) and the Col E1 origin of replication (from pJB83-DT9A).

Please amend the paragraphs on page 158, line 29 to page 160, line 2, as follows:

A 69-bp DNA fragment containing DNA encoding the human IL-2 signal peptide was obtained through nucleic acid amplification, using appropriate primers for IL-2, of an HEK 293 cell line (see, *e.g.*, U.S. Patent No. 4,518,584 for an IL-2 encoding DNA; see, also SEQ ID No. 9; the IL-2 gene and corresponding amino acid sequence is also provided in the Genbank Sequence Database as accession nos. K02056 and J00264). The signal peptide includes the first 20 amino acids shown in the translations provided in both of these Genbank entries and in SEQ ID NO. 9. The corresponding nucleotide sequence encoding the first 20 amino acids is also provided in these entries (see, *e.g.*, nucleotides 293-52 of accession no. K02056 and nucleotides 478-537 of accession no. J00264), as well as in SEQ ID NO. 9. The amplification primers included an *Eco*RI site (GAATTC) for subcloning of the DNA fragment after ligation into pGEMT (Promega). The forward primer is set forth in SEQ ID No. 11 and the sequence of the reverse primer is set forth in SEQ ID No. 12.

TTTGAATTCATGTACAGGATGCAACTCCTG forward (SEQ ID No. 11)
TTTGAATTCAGTAGGTGCACTGTTTGTGAC [reverse] reverse (SEQ ID No. 12)

b. Preparation of the *R. reniformis* luciferase-encoding DNA

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The initial source of the *R. reniformis* luciferase gene was plasmid pLXSN-RUC. Vector pLXSN (see, *e.g.*, U.S. Patent Nos. 5,324,655, 5,470,730, 5,468,634, 5,358,866 and Miller *et al.* (1989) *Biotechniques* 7:980) is a retroviral vector capable of expressing heterologous DNA under the transcriptional control of the retroviral LTR; it also contains the neomycin-resistance gene operatively linked for expression to the SV40 early region promoter. The *R. reniformis* luciferase gene was obtained from plasmid pTZrLuc-1 (see, *e.g.*, U.S. Patent No. 5,292,658; see also the Genbank Sequence Database accession no. M63501; and see also Lorenz *et al.* (1991) *Proc. Natl. Acad. Sci. U.S.A.* 88:4438-4442) and is shown as SEQ ID NO. 10. The 0.97 kb *EcoRI/SmaI* fragment of pTZrLuc-1 contains the coding region of the *Renilla* luciferase-[encoding] encoding DNA. Vector pLXSN was digested with and ligated with the luciferase gene contained on a pLXSN-RUC, which contains the luciferase gene located operably linked to the viral LTR and upstream of the SV40 promoter, which directs expression of the neomycin-resistance gene.

Please amend the paragraph on page 169, line 15, to page 170, line 2, as follows:

One set of control transgenic mice was generated by microinjection of mouse embryos with the β -galactosidase gene alone. The microinjection procedure used to introduce the plasmid DNA into the mouse embryos is as described in Example 13, but modified for use with embryos (see, *e.g.*, Hogan *et al.* (1994) *Manipulating the Mouse Embryo, A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, see, especially pages 255-264 and Appendix 3). Fertilized mouse embryos (Strain CB6 obtained from Charles River Co.) were injected with 1 ng of plasmid pCH110 (Pharmacia) which had been linearized by digestion with *Bam*HI. This plasmid contains the β -galactosidase gene linked to the SV40 late promoter. The β -galactosidase gene product provides a readily detectable marker for successful transgene expression. Furthermore, these control mice provide confirmation of the

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microinjection procedure used to introduce the plasmid into the embryos. Additionally, because the megachromosome that is transferred to the mouse embryos in the model system (see below) also contains the β -galactosidase gene, the control transgenic mice that have been generated by injection of pCH110 into embryos serve as an analogous system for comparison of heterologous gene expression from a plasmid versus from a gene carried on an [artificial] artificial chromosome.

Please amend the paragraph on page 176, lines 18-27 as follows:

Mammalian artificial chromosomes, such as the SATACs and minichromosomes described herein, can be modified to incorporate detectable reporter genes and/or transgenes of interest for use in developing transgenic chickens. Alternatively, chicken-specific [artificial] artificial chromosomes can be constructed using the methods herein. In particular, chicken artificial chromosomes (CACs) can be prepared using the methods herein for preparing MACs; or, as described above, the chicken [libraries] libraries can be introduced into MACs provided herein and the resulting MACs introduced into chicken cells and those that are functional in chicken cells selected.

Please amend the paragraph on page 179, lines 19-24, as follows:

Alternatively, the artificial chromosomes may be introduced into chick zygotes, for example through direct microinjection (see, *e.g.*, Love *et al.* (1994) *Biotechnology* 12:60-63), which thereby are incorporated into at least a portion of the cells in the chicken. Inclusion of a tissue-specific promoter, such [an] an egg-specific promoter, will ensure appropriate expression of operatively-linked heterologous DNA.

IN THE CLAIMS:

Please amend claims 43 and 59 as follows;

43. A method for producing a plant artificial chromosome, comprising:
introducing a DNA fragment into a plant cell, wherein the DNA fragment comprises a selectable marker;

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growing the cell under selective conditions to produce cells that have incorporated the DNA fragment into their genomic DNA;

selecting from among those cells a cell that has produced a dicentric chromosome; and

growing that cell under selective conditions, whereby a cell that contains a chromosome comprising a heterochromatic arm is produced.

59. A method of producing a transgenic plant, comprising:

introducing a DNA fragment into a first cell, wherein the DNA fragment comprises a selectable marker;

growing the cell under selective conditions to produce cells that have incorporated the DNA fragment into their genomic DNA; and

selecting a cell that comprises a satellite artificial chromosome (SATAC); and

isolating the SATAC and introducing it into a plant[or animal] cell.

* * *

Respectfully submitted,
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